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A method for single pair mating in an obligate parasitic nematode

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ABSTRACT

Parasitic nematodes species have extremely high levels of genetic diversity, presenting a number of experimental challenges for genomic and genetic work. Consequently, there is a need to develop inbred laboratory strains with reduced levels of polymorphism. The most efficient approach to inbred line development is single pair mating, but this is challenging for obligate parasites where the adult sexual reproductive stages are inside the host, and so difficult to experimentally manipulate. This paper describes a successful approach to single pair mating in a parasitic nematode, *Haemonchus contortus*. The method allows for polyandrous mating behaviour and involves the surgical transplantation of a single adult male worm with multiple immature adult females directly into the sheep abomasum. We used a panel of microsatellite markers to monitor and validate the single pair mating crosses and to ensure that the genotypes of progeny and subsequent filial generations were consistent with those expected from a mating between a single female parent of known genotype and a single male parent of unknown genotype. We have established two inbred lines, that both show a significant overall reduction in genetic diversity based on microsatellite genotyping and genome-wide single nucleotide polymorphism (SNP). There was an approximately 50% reduction in heterozygous SNP sites across the genome in MHco3.N1 line compared to the MoHco3(ISE) parental strain. The MHco3.N1 inbred line has subsequently been used to provide DNA template for whole genome sequencing of *H. contortus*. This work provides proof of concept and methodologies for forward genetic analysis of obligate parasitic nematodes.

1. Introduction

Parasitic nematodes are amongst the most important pathogen groups causing quality of life threatening disease in humans worldwide (Prichard et al., 2012). Approximately 2.0 billion people, mostly living in impoverished regions where sanitation is poor, are affected by soil-transmitted helminthiasis. These diseases result in an array of clinical effects, ranging from gastrointestinal disorders to anaemia, reduced physical fitness, decreased cognitive function and poor growth (De Silva et al., 2003). The control of human soil-transmitted helminthiasis is underpinned globally by the use of anthelmintics in mass drug administration (MDA) programmes (McCarty et al., 2014; Supali et al., 2013; Harris et al., 2015). Nematode parasites are also important causes of production limiting diseases in ruminant livestock (Nieuwhof and Bishop, 2005), being particularly relevant in already impoverished subtropical regions (Besier et al., 2016). The use of anthelmintic drugs had led to the selection of drug resistance, which is now widespread in many parasites grazing livestock. Concerns are now emerging regarding similar problems for the MDA programs being used to control human helminths.

There is heritable variation in traits such as virulence, host-specificity, environmental adaption and drug resistance, which are important constraints to sustainable helminth control in both humans and animals (Criscoine et al., 2009). There is a huge potential to use genetic crossing and mapping approaches in parasitic helminths to identify both single and quantitative trait loci underlying phenotypes of interest (Chevalier et al., 2014). For example, genetic crossing experiments are more powerful in identifying loci associated with anthelmintic resistance than laboratory selection, or the study of specific candidate genes chosen largely on the basis experimental work implicating them as encoding drug targets, or molecules involved in drug efflux (Rezanoff et al., 2016). However, although genomic resources are rapidly advancing with genome projects being undertaken for an increasing number of species, we still lack the basic tools and techniques to undertake genetic crossing and mapping in parasitic nematodes. Undertaking genetic crosses requires genetically and phenotypically divergent, and preferably near-isogenic parental lines. Developing these for nematode parasites is challenging since they cannot be maintained *in vitro* throughout their life cycle, hence experimental models depend upon the infection of the parasites' mammalian hosts. High levels of host specificity make these models intractable for human parasites and necessitate the development of animal models. The ruminant nematode parasite, *H. contortus* is currently the most important model system for the study of anthelmintic drug resistance as well being a key tool for anthelmintic drug and vaccine discovery research (Gilleard, 2013). A draft genome sequence has recently been published (Laing et al., 2013) and assembly and annotation improvements are on-going (Laing et al., 2016). In common with a number of other parasitic

nematode species, the high level of genetic polymorphism has made high quality assemblies difficult to produce (Gilleard and Redman, 2016). Hence, the development of inbred parasitic nematode lines with reduced levels of sequence polymorphism is an important goal both for genome assembly and for undertaking genetic crossing and mapping approaches.

Parasitic nematodes are dioecious, sexually reproducing, diploid organisms, hence the conceptually simplest method of generating inbred lines is by single pair matings. However, there are no published reports of genetically validated single pair matings for any obligate parasitic nematode species to date. This paper describes successful single pair mating for *H. contortus* following direct transplantation of a single sexually immature adult male with multiple sexually immature adult females of the MHco3(ISE) strain into the abomasum of a recipient parasite-free sheep. The method exploits the ease of establishment of parasite populations in a host, while accounting for polyandry (Redman et al., 2008a). Genetic and genomic characterisation of the parental and derived inbred lines demonstrate that the procedure significantly reduced genetic polymorphism of the MHco3(ISE) reference genome strain. This provides proof of concept of single mating in obligate parasitic nematodes, opening up new avenues of genetic approaches to study the biology of these important organisms.

2. Materials and Methods

2.1. Parasite material

Cryopreserved L₃ larvae of the ISE strain (Otsen et al., 2000; 2001) were obtained from Dr Fred Borgsteede (Central Veterinary Institute, Lelystad, Netherlands) by J. S. Gilleard. The strain was subsequently maintained at the Moredun Research Institute by serial passage through donor sheep and renamed MHco3(ISE) to distinguish it from versions of ISE strain used in other laboratories (Redman et al., 2008b). This strain was adopted as the original reference strain for the *H. contortus* genome project being undertaken at the Wellcome Trust Sanger Institute (Laing et al., 2013).

2.2. Genetic crossing by surgical transplantation

The overall experimental scheme is shown schematically in Fig. 1. To produce sexually immature adults for surgical transfer, a four month-old 'worm-free' donor lamb was orally dosed on day 0 with approximately 10,000 MHco3(ISE) *H. contortus* L₃. The donor lamb was euthanased on day 14 post-infection and the contents of its abomasum were collected. A single sexually immature male and 32 sexually immature female *H. contortus* L₄ were then surgically transferred, within 2

hours of recovery from the donor lamb, into the abomasum of a 4 month-old recipient lamb (lamb A, Fig. 1). In addition, a single sexually-immature male and 20 sexually-immature females were surgically transferred to two other lambs (lambs B and C, Fig. 1). The faecal trichostrongyle egg counts (FEC) of the three recipient lambs were monitored daily from days 14 to 21 (1 to 7 days post transplantation) using a standard salt floatation method with a minimum detection threshold of 1 egg per gram (epg) (Christie and Jackson, 1982). All three lambs had positive egg counts by day 18 that increased to 20 epg on day 21.

2.3. Collection of progeny from single female adult nematodes following mating with a single male

The three recipient lambs were euthanased 7 days after surgical transfer (day 21). The single, transplanted male worms could not be recovered from any of the three recipient lambs at autopsy but 12, 6 and 12 of the transplanted female worms were recovered from the abomasa of recipient lambs A, B and C, respectively. All recovered female worms were immediately picked into sterile phosphate buffered saline (PBS) and transferred individually into separate wells of 24 well plates each containing 1 ml of warm RPMI 1640 cell culture media (Gibco) and incubated in 5% CO₂ at 37°C for 8 hours to promote egg shedding. They were then transferred to a 24°C incubator for 36 hours to permit hatching of any fertilised eggs. Although all of the recovered female worms shed several hundred eggs, the development and hatching rate was extremely low. Consequently, only 4 female MHco3 *H. contortus* produced L₁ broods of sufficient size (minimum n = 100) to allow their molecular and phenotypic characterisation and subsequent propagation of another generation. These four females were arbitrarily named N1 (recovered from recipient lamb A), N2, N3 and N4 (recovered from recipient lamb B) respectively. DNA lysates were prepared from approximately half of the L₁ stage larvae in each brood (F₁ progeny of the single parent mating) and from the head of each adult female parent. The remaining L₁ were retained for coproculture development to the infective L₃ larval stage, to allow infection of more animals to produce the next filial generation.

2.4. Larval coproculture

L₁ were either transferred onto a disc of cotton filter paper placed in a petri dish containing 5 ml of an OP50 *Escherichia coli* culture in Luria broth/streptomycin, or inoculated into 10 g of faeces collected from a known 'worm-free' donor lamb that had been sequentially treated with 5 mg/kg of fenbendazole and 7.5 mg/kg of levamisole 5 days previously. The filter paper/*E. coli* and larval coprocultures were then placed individually in perforated plastic bags and incubated in a closed laboratory incubator at 24°C for 7 days. L₃ were then recovered from the larval cultures by

Baermannisation (MAFF, 1986) and transferred in tapwater into tissue culture flasks and stored at 8°C for 3 weeks before they were used to infect donor lambs. 15, 20, 3 and zero L₃ were recovered from the coprocultures of the broods of the adult female MHco3 individuals, N1, N2, N3 and N4.

2.5. Propagation of inbred lines resulting from single pair matings

In order to propagate filial lines, two 6 to 7 month-old 'worm-free' lambs were orally infected with 15 MHco3.N1.F₁ L₃ larvae (lamb D, Fig. 1) and 20 MHco3.N2.F₁ L₃ (lamb E, Fig. 1). The FECs of these two lambs were monitored from 14 days post-infection. Mean daily FECs were 1.2 (SD, 1.0) epg (lamb D), and 22 (SD, 16) epg (lamb E) between 21 and 60 days post-infection (Fig. 1). In order to propagate the next filial generation, this process was repeated using L₃ larvae derived from eggs (F₂ progeny) recovered from lambs D and E (Fig. 1). Two different 8 to 9 month-old 'worm-free' lambs were infected separately with 7,500 MHco3.N1.F₂ (lamb F, Fig. 1) and MHco3.N2.F₂ L₃ (lamb G, Fig. 1). The mean daily FECs between 20 and 60 days post-infection of lamb F and lamb G were 547 (SD, 265) epg and 420 (SD, 200) epg, respectively. An additional round of passage was undertaken for the MHco3.N1 line only. 7,500 and 5,000 MHco3.N1.F₃ L₃ derived from eggs recovered from lamb F were used to infect a 10 month-old lamb to produce a MHco3.N1.F₄ (lamb H, Fig. 1) lines. The mean daily FECs of this lambs between 20 and 60 days post-infection was 33 (SD, 29) (Fig. 1).

Freedom of contamination of the *H. contortus* populations with other nematode species was periodically tested during the development of the inbred lines by fluorescent agglutinin staining (Palmer and McCombe, 1996) and examination of larval morphology (Van Wyk and Mayhew, 2013). The lambs were maintained for between 2 and 5 months before they were euthanased and any surviving *H. contortus* were recovered from their abomasa, counted and stored in 70% ethanol.

2.6. DNA Lysate preparation

Individual worm DNA lysates were prepared from female heads, L₁ (F₁) and sodium hypochlorite-exsheathed L₃ (F₂, F₃ and F₄) in a volume of 25 µl using standard techniques (Redman et al., 2008b). Bulk worm preparations of 500 ex-sheathed L₃ were made for F₂, F₃ and F₄ generations. 1 µl of a 1:30 dilution of female head lysates or of a 1:10 dilution of L₁ lysates was used as PCR template. Dilutions of lysate buffer without template, made in parallel, were included as negative controls for all PCR amplifications. All DNA lysates were subjected to a previously published ITS-2

rDNA PCR assay, to confirm species identity as *H. contortus* (Redman et al., 2008b; Wimmer et al., 2004).

2.7. Single strand conformation polymorphism

The genetic diversity of the GABA Cl subunit HG1 locus (Blackhall et al., 2003), as well as that of GluCl α and β subunit loci (Blackhall et al., 1998) (Supplementary Fig. S2A) was examined by single strand conformation polymorphism (SSCP) using previously described PCR primers (Blackhall et al., 2003). The thermal cycler conditions used were: 95°C for 4 minutes; followed by 40 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds; a final extension stage of 72°C for 5 minutes. Amplicons were first visualised on a 1.2% agarose gels and then run on non-denaturing polyacrylamide gels as previously described (Skuce et al., 2010).

To confirm the allelic assignments of the GABA Cl subunit HG1 SSCP genotyping, the PCR products produced from two individual MHco3.N1.F₂ L₃ DNA heterozygotes (6F and 9G) were cloned and sequenced. Briefly, amplicons were run on a 1% agarose electrophoretic gel to enable the excision of 305 bp bands from which DNA was then isolated (QIAquick Gel Extraction kit, Qiagen). DNA was ligated into pGEM®-T (Promega) plasmid vectors to allow transformation into JM109 competent *E. coli* cells (Stratagene). Plasmid DNA from the cultured transformed cells was then purified using a Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced using SP6 and T7 universal primers in both orientations.

2.8. Microsatellite genotyping

Microsatellite genotyping of 'bulk' DNA lysates, made from approximately 500 larvae, was performed on MHco3(ISE), MHco3.N1.F₂, MHco3.N1.F₃, MHco3.N1.F₄, MHco3.N2.F₂ and MHco3.N2.F₃ *H. contortus* lines. Ten microsatellite markers, previously shown to be polymorphic in the MHco3(ISE) strain were used: Hcms25, Hcms33 and Hcms36 (Otsen et al., 2001), Hcms8a20, Hcms22co3 (Redman et al., 2008b), HcmsX142, HcmsX256, HcmsX337 (Redman et al., 2008a), Hcms3561 and Hcms18210 (Redman et al., 2012). Individual worm genotyping was also performed for four of these loci, Hcms8a20, Hcms36, Hcms3561 and Hcms25 plus two additional loci, HcmsX182 and HcmsX240 (Redman et al., 2008a), on the N1 and N2 adult female parent heads and 30 individual larvae for each of the following populations: MHco3.N1.F₁ and MHco3.N2.F₁ (L₁); MHco3.N1.F₂, MHco3.N2.F₂, MHco3.N1.F₃, MHco3.N2.F₃ (L₃) and MHco3(ISE). All microsatellite genotyping, on both 'bulk' and single worm DNA lysates, was performed using the same PCR

amplification methods and parameters as previously described (Redman et al., 2008b). Capillary electrophoresis was performed using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) for the accurate sizing of microsatellite PCR products. The forward primer of each microsatellite primer pair was 5'-end labelled with FAM, HEX, or NED fluorescent dyes (MWG) and electrophoresed with a GeneScan ROX 400 (Applied Biosystems) internal size standard. Individual chromatograms were analysed using Genemapper Software Version 4.0 (Applied Biosystems).

2.9. Genetic analysis

Multilocus genotype principal coordinates analysis was conducted using GenAlEx version 6.1 add-in software (Peakall and Smouse, 2006) for Microsoft Excel to provide a schematic indication of the degree of inbreeding. The average number of alleles per locus, observed heterozygosities (H_o), and unbiased estimates of expected heterozygosity (H_e) were calculated using Arlequin version 3.11 software (Nei, 1978, Excoffier et al., 2005). Data were defined as 'standard' rather than 'microsatellite' because the loci did not adhere to the stepwise mutation model. Exact tests for Hardy-Weinberg equilibrium were tested per locus using Fisher's exact probability test based on contingency tables (Raymond and Rousset, 1995), where P-values <0.05 were taken as evidence of significant deviation. Significance levels were estimated using 100,000 Markov chain steps. Pairwise linkage disequilibrium was tested for using a likelihood-ratio test (Slatkin and Excoffier, 1996). For each locus, estimates of inbreeding (F_{is}) were calculated using an algorithm based on the formula ($H_e - H_o$) / H_e . Pairwise F_{st} values were calculated using Arlequin version 3.11 software. Analysis of Molecular Variance (AMOVA) was performed to test for population differentiation of samples at various levels, locus by locus using the Arlequin version 3.11 software.

2.10. Genome-wide SNP analysis

Genomic libraries were prepared from 400 MHco3(1SE) and 400 MHco3.N1.F₃ adult worms for Illumina sequencing using previously described methods (Laing et al., 2013; Kozarewa et al., 2009) (Supplementary Table S3). Preliminary analysis and base-calling for data from the Illumina HiSeq sequencing machines used the RTA1.8 analysis pipelines. Whole-genome shotgun sequence data was generated from these libraries on two different Illumina platforms, producing different numbers of reads and reads of different lengths (Supplementary Table S3). To produce comparable data between the two biological samples, read pairs were randomly sampled from the larger (inbred material) sequencing data by keeping each pair of reads in the subsampled file with a probability of 0.39, and by clipping 12 bp from each end of every read. Reads were mapped against the released

370Mb v1.0 genome assembly of *H. contortus* (Laing et al., 2013), available at the GenBank database under project ID PRJEB506, using the mapper SMALT v0.7.0.1 (<http://www.sanger.ac.uk/resources/software/smalt>) in paired-end mode, with an indexing k-mer size of 13 and step size of 1, mapping non-repetitively (-r -1), with a minimum identity of 0.8 to report a mapping (-y 0.8) and exhaustively searching for alignments of each read independently of its mate pair (-x), and only reporting reads as properly paired if they were mapped than 1000bp apart on the reference genome. Single nucleotide polymorphism (SNP) variants were called jointly from the three mapping output files using samtools v0.1.19-44428cd (Li et al., 2009) using the *mpileup* command, skipping alignments with either mapping or base quality scores less than 13. Density, distribution and types of variant calls were tallied using vcftools v0.1.11 (<https://sourceforge.net/projects/vcftools/>). Estimates of the nucleotide diversity (π) for the pools of worms sequenced in each library were calculated independently from the variant calling approach outlined above using PoPoolation2 v1.013 (Kofler et al., 2011).

2.11. Ethics statement

All experimental procedures described in this manuscript were examined and approved by the Moredun Research Institute Experiments and Ethics Committee and were conducted under approved UK Home Office licenses in accordance with the Animals (Scientific Procedures) Act of 1986. The Home Office license numbers are PPL 60/03223 and PPL 60/03899 and experimental IDs for these studies were E06/58, E06/75 and E09/36.

3. Results

3.1. The establishment of two independent inbred lines by single pair mating of *H. contortus*.

Our preliminary experiments to replicate anecdotal reports suggesting that it might be possible that a single male and a single female *H. contortus*, when transferred directly into the abomasum of a 'worm-free' recipient sheep could survive for long enough to find each other, mate and shed eggs were unsuccessful, highlighting the severe biological limitations to this approach. Consequently, we developed a method in which a single immature male worm was transplanted with a number of immature female worms, then following mating, the female worms were recovered on autopsy, placed in individual wells of a 24 well plate and allowed to lay eggs (F_1 progeny) *in vitro*. Two of the recovered female worms, designated N1 and N2, produced broods of sufficient size and viability to enable the propagation of the next filial generations. 15 and 20 L_3 from

the N1 and N2 founding female parents, respectively, were used to orally infect two separate lambs to establish the MHco3.N1 and MHco3.N2 inbred lines (Fig. 1).

3.2. Validation of single pair mating and assessment of polymorphism of inbred lines by microsatellite genotyping

Bulk DNA genotyping with 10 microsatellite markers, on DNA prepared from pools of approximately 500 L₃ per population, was used to provide an initial assessment of the genetic diversity of the MHco3.N1 and MHco3.N2 inbred lines and indicate of the success of the single pair matings. The total number of alleles detected was reduced in both of the inbred lines relative to the founding MHco3(ISE) population with a greater loss of overall diversity in MHco3.N1. Across the 10 markers, a total of 28 alleles in the MHco3(ISE) population was reduced to 15 and 20 alleles in the derived MHco3.N1 and MHco3.N2 lines respectively. There was a loss of alleles at 7 out of the 10 loci in both cases (Supplementary Table S1).

The MHco3(ISE) strain, the individual N1 and N2 founder female parents and populations of the inbred MHco3.N1 and MHco3.N2 lines were analysed in more detail by genotyping individual worms at six of the most discriminatory loci. The level of polymorphism of the parental MHco3(ISE) strain was consistent with that previously observed with other panels of microsatellite loci (Redman et al., 2008b) with a mean of 3.17 alleles per locus and an expected heterozygosity (H_E) of 0.572. There was a clear reduction in polymorphism in F₁ and F₂ populations of both inbred lines with the MHco3.N1 again showing the greatest reduction (Supplementary Table S2).

Pairwise F_{ST} estimates based on the multi-locus genotype data revealed a high degree of genetic differentiation between the two inbred strains as well as between both lines and the parental MHco3(ISE) strain. No statistically significant genetic differentiation was observed between any of the filial populations within the same inbred line demonstrating that the genetic integrity of both the inbred lines was maintained despite passage (Supplementary Fig. S1A). Further evidence for reduction in genetic diversity by the single parent mating procedure was provided by principal component analysis of individual worm multi-locus genotypes (Supplementary Fig. S1B).

3.3. Examination of the HG1 GABA Cl locus using SSCP

The HG1 gene which encodes a GABA-gated chloride channel (Blackhall et al., 2003) was selected and used as an additional marker to monitor the single pair mating and inbreeding process, since this had been shown to have a high level of genetic diversity in the MHco3(ISE) strain

(Supplementary Fig. S2). SSCP profiles were obtained for 84 MHco3(ISE) L₃ and at least 15 distinct profiles were discernable (Supplementary Fig. S3A). However, only three different SSCP profiles were discernable from 57 F₁ and 65 F₂ progeny of the MHco3.N1 inbred line (Supplementary Fig. S3B).

3.4. Comparison of MHco3(ISE) and MHco3.N1 genome-wide SNP polymorphism

Whole genome Illumina sequencing was performed on MHco3(ISE) and MHco3.N1 populations (Supplementary Table S3). The number of sites classified as heterozygous within the MHco3.N1 population in the variant calls from *mpileup* was almost 50% fewer than those called for the MHco3(ISE) populations. Using the number of reads supporting each allele at a site as a rough estimate of the allele frequency in the pool of adult worms sequenced, there is a clear pattern of a greater proportion of sites having minor alleles segregating at intermediate frequencies (between 0.15 and 0.35) within the MHco3.N1 population (Supplementary Fig. S4A). The MHco3.N1 population is particularly reduced in rare alleles, as expected from a recent, extreme population bottleneck. This pattern is consistent with these nematodes being the offspring a single-pair mating, where we would expect minor alleles to be present on just one of the four parental haplotypes. The same pattern is clear in the subset of sites that are polymorphic in both populations, where the nucleotide diversity (π) is lower in MHco3.N1 than in MHco3(ISE) at almost two-thirds of sites (332/537) on the longest assembly scaffold (Supplementary Fig. S4B).

4. Discussion

The original ISE strain of *H. contortus* had been previously inbred from the outbred SE population (Otsen et al., 2000; 2001). This was achieved by dissecting the eggs from an adult female SE strain *H. contortus*, culturing these eggs for 7 days in 'worm-free' faeces, and then injecting recovered L₃ into the forestomach of recipient sheep. The recipient sheep were euthanased one week after they had started shedding trichostrongyle eggs. A single benzimidazole susceptible adult female *H. contortus* had then been selected on the basis of its β -tubulin isotype 1 genotype (Kwa et al., 1994), and the process repeated through fifteen generations (Roos et al., 2004), to yield what was considered to be an inbred benzimidazole susceptible isolate. However, genetic analysis with microsatellite markers subsequently revealed high levels of genetic polymorphism in the MHco3(ISE) strain of *H. contortus* (Redman et al., 2008b).

This paper presents the development of a novel method to achieve a single pair parasitic nematode mating involving the surgical transfer of multiple female and one male day 14 parasitic-

stage *H. contortus* to the abomasum of recipient lambs. The experimental protocol took into account the known polyandrous mating behaviour of *H. contortus* (Redman et al., 2008a) by using a single male transplanted with multiple females. The method exploited the ability to differentiate between male and female nematodes before they reach sexual maturity, which is a prerequisite for genetic crosses (Chevalier et al., 2016). The transplanted male worm successfully fertilised multiple female worms in each case and this experimental design ensured each female brood was from a single pair mating event. The experimental design also prevented any risk of extraneous parasitic nematode infection of recipient sheep by euthanasing them and recovering egg laying female *H. contortus* well within the minimum prepatent period of contaminant parasitic nematodes. Potential issues caused by fly-borne parasitic nematode contamination of coprocultures were addressed by their incubation in an isolated closed environment, primarily on filter paper in a live *E. coli* system. The methods used to prevent parasitic nematode contamination were apparently effective for the development of the MHco3.N1 and MHoc3.N2 lines, since the genetic analyses presented in this paper are consistent with those expected from a single pair mating event. Failure to recover the male parent *H. contortus* from any of the three recipient lambs, while between 30% and 60% of the females were recovered was disappointing. This could be due to chance, or might suggest that the behaviour of male parasitic nematodes in seeking out females predisposes to their loss from the abomasum. Determination of both parental genotypes founding the inbred lines would have aided further genetic validation based on the male parental genotype.

The microsatellite individual genotyping data was entirely consistent with that expected if the two MHco3.N1 and MHco3.N2 inbred lines, were founded by single pair matings of the N1 and N2 female parents. Although the lack of knowledge of the male parental genotypes precluded definitive Mendelian genetic analysis of the crosses, the data overall provided strong support of the success of the single pair matings. The appropriate maternal alleles for each microsatellite marker were present in the filial generations of each cross and the total number of alleles present was entirely consistent with single pair mating. The multilocus genotype analysis of MHco3.N1.F₁ and MHco3.N2.F₁ worms was also strongly supportive of successful single pair mating, with the F₁ multilocus genotypes forming tight clusters around the respective maternal parental genotypes on PCA plots.

An overall loss of genetic polymorphism in both the MHco3.N1 and MHco3.N2 lines compared with the parental MHco3 (ISE) strain was revealed by the microsatellite markers, the GABA CI SSCP profiles and the whole genome sequencing analysis. The Hco3.N1 line showed the greatest loss of polymorphism of the two inbred lines based on the microsatellite genotyping with a loss of 13 out of 28 alleles (46%) across the 10 microsatellite markers genotyped. Consequently, the

inbred MHco3.N1 population was propagated further and used as the reference strain for the *H. contortus*, genome project (Laing et al., 2013). Subsequent, genome-wide SNP analysis was consistent with the microsatellite analysis showing that MHco3.N1 line had an almost 40% reduction in SNP positions called as heterozygous across the genome, and reduced nucleotide diversity at shared heterozygous sites, compared to MHco3(ISE).

The two inbred lines retained largely different alleles from the MHco3(ISE) populations, as demonstrated in the multilocus genotyping PCA plots and pairwise F_{ST} analyses. The production of genetically divergent inbred *H. contortus* lines using this method could be exploited in a number of ways. It could be used to develop genetically divergent strains with which to undertake genetic crosses for the production of a genetic map, or to identify the position of genetic loci of interest, such as those underlying anthelmintic resistance (Le Jambre et al., 1999). A prerequisite for the creation of a genetic map is that the parent populations have minimal within-strain polymorphism, but high levels of between-strain polymorphism, in order to allow the alternative parental alleles to be identified in F_2 progeny resulting from the genetic cross. With the exception of the Chiswick avermectin resistant (CAVR) strain, which arose as a serendipitous, extraneous, ivermectin resistant contaminant of an Australian laboratory passaged *Trichostrongylus colubriformis* strain (Le Jambre, 1993), the currently available laboratory strains of *H. contortus* are too polymorphic to use in conventional mapping studies. Hence generation of experimentally inbred, near-isogenic, genetically divergent strains is useful. Cases of multigenic resistance could be investigated by segregating different genetic loci contributing to an anthelmintic resistance phenotype into separate inbred lines.

In conclusion, the proof of concept of molecular and genetic validation of a single parent mating method to inbreed *H. contortus* will provide a potentially useful tool in the further development of genomic resources that are needed to inform sustainable nematode parasite control.

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Contributions

NDS, JSG and FJ conceived the method. NDS, AAM, DJB and FJ undertook the animal work and gross parasitology. NDS, EM, HNv-G, NH, MB and JAC undertook the molecular studies and analysis of the data. NDS, ER, JAC and JSG wrote the paper.

Figure legend

Fig. 1. Genetic crossing and passaging approach to inbreed the MHco3(ISE) standard genome strain of *H. contortus*.

Schematic representation of experimental aim and summary of nomenclature.

*gDNA extracted from MHco3.N1.F3 adults used for genome sequencing (Laing et al., 2013)

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Supplementary data

Supplementary Fig. S1. Genetic differentiation between populations of inbred worms using a panel of four microsatellite markers.

(A) Population pairwise F_{ST} s comparing the F_1 and F_2 populations of the MHco3.N1 and MHco3.N2 inbred lines and the parent MHco3(ISE) population. Significant genetic divergence is highlighted by underlining and bolding.

The data show a high degree of genetic differentiation between the two inbred strains (pairwise F_{ST} = 0.300-0.512) as well as between both lines and the parental MHco3(ISE) strain. The MHco3.N1 line appears to be the slightly more divergent from the parental MHco3(ISE) strain than the MHco3.N2 line (eg. MHco3.N1. F_1 F_{ST} = 0.262 compared with MHco3.N2. F_1 F_{ST} = 0.197). The F_{ST} values remain statistically significant between the MHco3(ISE) population and both inbred lines at each filial generation. In contrast, no statistically significant genetic differentiation was observed between any of the filial populations within the same inbred line.

(B) Individual multi-locus genotypes of worms from Hco3(ISE), MHco3.N1. F_1 and MHco3.N2. F_1 populations shown on a PCA plot as black triangles, green diamonds and red squares respectively.

The individual founding N1 and N2 female parental multi locus genotypes are shown as a larger green diamond, or red square, respectively, and indicated by an arrow.

The individual worm multilocus genotypes of worms from the two inbred populations formed much tighter clusters than those from the founding MHco3(ISE) population. The clusters of for each inbred line were completely separate to each other and centred around the position of the multilocus genotype their respective N1 and N2 individual founder female parents on the PCA plot.

Supplementary Fig. S2. SSCP polyacrylamide gels.

(A) Gel comparing polymorphisms in GluCl α (lanes 1 – 4), GluCl β (lanes 6 – 9) and GABA Cl (lanes 11 – 14) subunits of four individual adult MHco3(ISE) *H. contortus* (1.5 mM MgSO₄ and 5 μ l of PCR product run on gel). Each of the MHco3(ISE) *H. contortus* has a different GABA Cl SSCP genotype.

(B) A polyacrylamide gel showing the GABA Cl SSCP genotypes of a MHco3(ISE) female head (lane 17) and L₃ progeny (lanes 1, 2, 5, 6, 7, 10, 11, 12, 13, 14, 15, 16 and 19) from eggs hatched to L₁ in RPMI in the well of a 24 well plate, and then grown to L₃ in the same well on *E. coli* and filter paper. Amplified PCR product was not seen on an agarose gel corresponding with lanes 3, 4, 8 and 9, while lane 18 is a negative control. Different SSCP genotypic profiles are labelled A – E.

Supplementary Fig. S3. GABA Cl subunit HG1 SSCP profiles.

(A) Polyacrylamide gels showing a high level of polymorphism in MHco3(ISE) *H. contortus*. Each lane shows the SSCP GABA Cl subunit HG1 genotype of an individual L₃.

(B) Polyacrylamide gels showing the GABA Cl SSCP genotypes of the N1 female (lanes 5, 15, 25 and 35) and of individual MHco3.N1.F₁ L₁ (lanes 1, 2, 4, 6 – 14, 16 – 19, 21, 23, 24, 26 – 32, 34, and 36 – 40).

(C) Polyacrylamide gels showing the GABA Cl SSCP genotypes of MHco3.N1.F₂ L₃.

Although the assignment of alleles from SSCP profiles is often ambiguous, the SSCP profile of the founding N1 female parent was entirely consistent with those of the F₁ and F₂ progeny (data not shown).

Supplementary Fig. S4. Genomic analysis of allele frequency and nucleotide diversity.

(A) Histogram of minor allele frequencies for MHco3(ISE) and MHco3.N1 inbred line based on genome sequencing data, based on counts of reads supporting each of two alleles at all biallelic sites in each sequencing library.

579 (B) Nucleotide diversity estimates for 537 sites on scaffold 1 of the *H. contortus* v1.0 assembly
580 polymorphic in both MHco3 (ISE) and MHco3.N1. The dashed line represents equal diversity in the
581 pools of worms from each population, the solid line is the best-fit linear regression through the
582 origin, with the 95% confidence interval for this fit shaded.

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